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Evaluating the impact of shade on nitrogen fixation in sulla (*Hedysarum coronarium* L.): a ^{15}N natural abundance study

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ABSTRACT

Growing legumes among trees in agroforestry systems (AFS) can increase farmland productivity reducing the dependence of farming systems on external non-renewable inputs, Such as mineral nitrogen (N) fertilizers. However, the crop-tree competition for resources limits legume growth due to a shortage of photosynthates and consequently N_2-fixation. Sulla (*Hedysarum coronarium* L.) is commonly grown in forage cropping systems in many areas of central Italy due to its adaptability to clayey soils and hilly conditions, and high nutritional value as hay or pasture. As a C3 species, sulla could be a suited for growth in sparse tree crop systems, such as olive orchards, that are typical in the Mediterranean basin. However, it is not well known if an increasing level of shade could affect the N_2-fixing performance of sulla. An artificial shade plot experiment was conducted for two years in a coastal plain area near Pisa, Italy, to simulate, under rainfed conditions, the shading provided by trees through diverse levels of light reduction. Shade treatments were applied through wood slat structures inducing three increasing levels of light reduction: full-sun (no shade), 30 % (moderate shade) and 50 % (high shade). The isotopic method based on ^{15}N natural abundance was used to estimate the biological nitrogen fixation of sulla. The total N yield and N fixed were higher in the full sun plots (+55 %), while a higher % of N derived from the atmosphere (%Ndfa) was observed in the intensive shade plots, especially in late spring (+50 %). These trends can be explained by the reduced biomass production caused by intense shading. The relationships between % Ndfa and limited light availability showed that sulla could be a suitable forage crop for AFS, in particular, when exposed to moderate shade. Further research is necessary to assess the ecophysiological implications involved in the N_2-fixation process of sulla and other legume crops under AF conditions, as well as the eventual N-transfer to the intercropped trees.

1. Introduction

Agroforestry (AF) can be defined as the integration on the same unit of land of trees and crops, and/or animals, in interactive combinations to provide a range of ecosystem services and benefits (Ramachandran Nair et al., 2010; Kim and Isaac, 2022). For example, it can enhance land productivity by increasing overall biomass production compared to specialized crops (Querné et al., 2017). Furthermore, AF is considered to be one of the agricultural practices potentially more suited to mitigation of the effects of climate change (Kay et al., 2019). This is because agroforestry systems (AFS) can enhance the complexity of agricultural landscapes, while allowing higher crop production, reduction of greenhouse gas (GHG) emissions, soil and water losses, improving biodiversity conservation, soil fertility and carbon sequestration. (Quinkenstein et al., 2009; Ramachandran Nair et al., 2010; Smith et al., 2012; Wezel et al., 2014; Nasielski et al., 2015; Torralba et al., 2016; Muchane et al., 2020; Kim and Isaac, 2022). Furthermore, AFS can buffer extreme weather conditions at the farm/field microclimatic scale by reducing wind speed and both temperature and humidity oscillations close to the soil surface (Wezel et al., 2014; Jo and Park, 2017; Panozzo et al., 2022). However, there are also critical challenges and barriers for a wider adoption of AF, such as the necessary expertise and know-how (Tranchina et al., 2024), high early costs in terms of both finance and labour demand that do not offer instant profitability

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for farmers (Kim and Isaac, 2022), and the absence of strong policy instruments to support farming transition to AF (Ndlovu and Borrass, 2021).

Besides the many agroecosystem services delivered by AFS, it is still unclear as to how AFS could help reduce the reliance of cropping systems on mineral nitrogen (N) fertilizers, that is one of the key targets in Europe according to the Farm to Fork strategy. This is a relevant aspect in the Mediterranean area, where N_2-fixing tree crops are rarely grown, and trees grown in AFS may require high level of N inputs. Hence, introducing N_2-fixing herbaceous crops among trees may improve N cycling, thus reducing the need for mineral N fertilizers at agroecosystem level (Querné et al., 2017). The amount of N fixed by legumes could be close to, or even higher than the usual application rates of N fertilizers (Unkovich et al., 2010). For example, in Central Italy, sulla (*Hedysarum coronarium* L.) is a legume species traditionally grown as a biennial forage crop, especially for sheep grazing, due to: (i) its ability to thrive in clay soils even under dry conditions, (ii) its adaptability to alkaline soils, and (iii) its high nutritional value for ruminants (Mantino et al., 2020). Therefore, it could be a suitable species to be introduced into AFS.

In AF experiments conducted in N limiting conditions (in soils with low N content where non-legume trees outcompete the companion herbaceous crops for soil N), legumes can invest more energy and resources (e.g., photosynthates) to support biological N_2-fixation (Tramacere et al., 2023). Indeed, under N deficiency conditions, legume crops and trees can invest higher energy to support nodulation and Biological Nitrogen Fixation (BNF) without, rather than with, the application of N fertilization (Wanek and Arndt, 2002; Isaac et al., 2014). However, tree competition for essential resources such as light, water and nutrients (Isaac et al., 2014; Nasielski et al., 2015) could curb the growth of legumes species and, consequently, also N_2-fixation due to a shortage of resources to sustain rhizobial activity (Querné et al., 2017).

According to the recent literature, several experiments have tested the effects of the shade of trees on the yield of legume crops (e.g., Lin et al., 1998; Koukoura et al., 2009; Kyriazopoulos et al., 2013; Ehret et al., 2015; Pang et al., 2019; Sanna et al., 2019; Mantino et al., 2021; Tramacere et al., 2024), and investigated the magnitude of tree-crop interactions at different inter-distances (Thevathasan and Gordon, 2004; Manceur et al., 2009; Peng et al., 2009; Rivest et al., 2009). On the other hand, few investigations have been conducted to assess the competitive mechanisms affecting growth and BNF of perennial legumes in AFS to improve the resource use efficiency and design systems that can facilitate positive interactions among plant species (Eichhorn et al., 2006; Carranca et al., 2015; Querné et al., 2017; Tramacere et al., 2023).

Normal light intensity can facilitate the symbiosis that occurs between *Rhizobia* bacteria and legumes by enhancing the photosynthetic capacity and improving the nutritional status of legume crops (Carranca et al., 2015). A continuous supply of carbohydrates is needed for Rhizobial bacteria in the symbiotic nodules to produce the required energy for their activity: i.e, to capture the atmospheric N_2 and transform it in reactive forms useful for plant metabolism (i.e., NH_4^+) (Carranca, 2013). In AFS the tree canopy can affect not only the quantity, but also the quality of light reaching understory vegetation in a limited area (Dubbert et al., 2014). Therefore, the competitiveness of legumes in AFS is affected by their capability to intercept the solar radiation, which has been identified as the most limiting factor for legumes growth (Cubera et al., 2009; Carranca et al., 2015). Without the availability of mature AFS sites with adult trees, an alternative method to evaluate shade tolerance is to simulate shading conditions. This could be done, for instance, by installing artificial shade structures on top of the canopy of herbaceous crops grown without companion trees and evaluating them under diverse levels of shade. According to the study of Varella et al. (2011), woody slat structures can more effectively simulate the shade of tree canopy compared to other methods, such as shade cloth, because they mimic the periodic light variation occurring in the presence of trees.

This study aimed to assess the effect of several levels of simulated shade on the N_2-fixing capability of sulla in Mediterranean rainfed conditions. For BNF assessment, the isotopic method based on ^{15}N natural abundance was used (Peoples et al., 2015). Our hypothesis was that a moderate level of shade would not reduce the quantity of N obtained from BNF in sulla, as the crop might compensate for the reduced biomass production caused by lower light availability with an increased rate of BNF.

2. Materials and methods

2.1. Site description and experimental layout

As reported in Tramacere et al. (2024), a field experiment was conducted at the Center for Agri-Environmental Research "Enrico Avanzi" of the University of Pisa (CiRAA), San Piero a Grado, Pisa, Italy (43°41′05.92″N, 10°20′31.34″E, 1 m above sea level and 0 % slope) from 2019 to 2022 under rainfed conditions. The experiment spanned two consecutive growing seasons of sulla and was replicated over time in two adjacent fields. Crops were cultivated for two consecutive years to observe their entire growth cycle: Experiment 1 covered the biennial cycle 2019–2021, while the Experiment 2 covered the cycle 2020–2022. Average annual rainfall and annual mean air temperature were, respectively, 892 mm and 14.9 °C (based on long-term data, from 1993 to 2021). The soil texture of the two fields measured in the 0–0,30 m layer was loam (18.67 % of clay, 46.37 % of silt, 34.96 % of sand) according to USDA (Soil Survey Stuff, 1999), with a pH value of 8.21, 2.40 % of organic matter content (Walkley–Black, 1934), 1.29 g kg^{-1} total nitrogen content (Bremner, 1960), 7.30 ppm of available P_2O_5 content (Olsen, 1954). The experimental layout for both fields was arranged as a completely randomized design with four replicates, with a plot size of 18 m^2 (6 m long per 3 m wide). The crops grown were sulla (SUL) cv. Silvan, and Italian ryegrass (*Lolium multiflorum* Lam.) (RYE) cv. Teanna. Italian ryegrass was cropped close to sulla plots, undergoing the same experimental treatment, aiming to be the reference crop for computing the Nitrogen derived from atmosphere. The experimental factor for the present study was shade, with three increasing levels of simulated shade: (i) no shade (NS), representing full light availability, (ii) moderate shade (MS) with a 30 % reduction in potential light availability, and (iii) high shade (HS) with a 50 % reduction of potential light availability. On shaded plots (MS and HS), shade was provided soon after sowing by using woody slats constructed based on the methodology outlined by Varella et al. (2011) and reported in detail in our previous study (Tramacere et al., 2024). The shade intensity levels applied were determined based on data from Mantino et al. (2021) regarding olive tree (*Olea europaea* L.) agroforestry systems. In Tuscany, olive trees, which are evergreen, are among the most widespread crops and are traditionally grown in low-density systems with scattered trees or frequently integrated into agroforestry systems (Paris et al., 2019).

2.2. Crop management

In both years, moldboard ploughing was performed at 0.3 m depth at the end of August on one of the two adjacent experimental fields. In September, triple superphosphate (0-46-0) was broadcast applied to supply 100 kg ha^{-1} of P_2O_5. Rotary harrowing was carried out to prepare the seedbed on 20th October 2019 (Experiment 1) and 10th November 2020 (Experiment 2). The sowing was performed using a mechanical plot seeder on 21st October 2019 for Experiment 1 and on 11th November 2020 for Experiment 2. The seeding rates were as follows: (i) sulla, 38 kg ha^{-1} and (ii) ryegrass, 30 kg ha^{-1}, with an inter-row distance of 0.15 m for both crops. Sulla seeds were not inoculated with rhizobial strains due to its frequent cultivation on those soils.

2.3. Data collection and chemical analysis

The meteorological data were collected using a public weather station (43°40′51.60″N, 10°16′48.00″E) operated by the Tuscany Regional government (http://www.sir.toscana.it). Prior to sowing, soil characteristics were assessed by sampling two pooled soil cores (0–30 cm soil layer) per plot using an auger. To simulate animal grazing, herbage mass was manually mowed during the growing season, specifically seven times in Experiment 1 and five times in Experiment 2, following the growing rate of the crop. In Experiment 1, the exact mowing dates were: 26th February 2020 (M1), 15th April 2020 (M2), 20th May 2020 (M3), 23rd June 2020 (M4), 18th November 2020 (M5), 31st March 2021 (M6), and 20th May 2021 (M7). In Experiment 2, the plots were mowed on: 14th April 2021 (M1), 27th May 2021 (M2), 24th June 2021 (M3), 12th November 2021 (M4), and 3rd May 2022 (M5). At each mowing date, the herbage mass was sampled on two areas of 0.25 m^2 per plot. The biomass of the forage crops was separated from that of the weeds and then fresh weighed, and oven-dried at 60 °C until a constant weight was achieved. Furthermore, at the end of each vegetative season (i.e., in September, after the first rainfall), the roots were also collected by means of a spade that was used to extract two undisturbed soil samples (pooled) per plot, covering the 0–30 cm soil layer and a width of 20 cm. The samples were carefully washed with water and the taproots from individual plants were then separated, counted and oven-dried at 60 °C.

To assess the level of N_2-fixation of the legume crop, homogenous grounded biomass samples (3–6 mg) were loaded in tin capsules, wrapped and weighted. The elemental and isotope analyses were carried out using an elemental analyzer (Vario Micro Cube, Elementar, Langenselbold, Germany) coupled with an isotopic ratio mass spectrometer (IsoPrime 100, Elementar, Manchester, UK), to measure N content (N %) and ^{15}N atom excess compared to the atmosphere (δ ^{15}N ‰), following the methodology of Peoples et al. (2015).

Additionally, at each harvest date, crop light availability was measured using a SunScan (Delta-T Devices Ltd, Cambridge, UK) multiprobe sensor placed under the slats (just below the slats, 80 cm above the ground). The SunScan probe and beam fraction sensor was calibrated to provide Photosynthetically Active Radiation (PAR) readings to evaluate if the slat structures were delivering the appropriate level of shade. On average, the percentage of PAR reduction compared to NS condition, measured under the slats over the three years, was approximately: (i) 30 % for MS, and (ii) 50 % for HS treatments.

2.4. Calculations

Roots dry matter (g m^{-2} of DM) was calculated as follows:

$$\text{Root DM} = \frac{\text{PCNR} \times \text{RDM}}{\text{RSNR}} \quad (1)$$

where PCNR is the count of emerged plants per square meter; RDM is the total dry weight of the taproots collected in each plot; RSNR is the number of taproots sampled in each plot on an area of 0.18 m^{-2} (reported to the area of 1 m^2).

Nitrogen yield (Nyield) (g m^{-2} of N) was calculated by multiplying the dry matter yield (DM) (g m^{-2}) of each single component of plant biomass (shoots and roots) per its total N content (Ntot %). According to ^{15}N natural abundance method reported in Peoples et al. (2015), the proportion of N derived from the atmosphere (%Ndfa) in legume shoots and roots was estimated using the equation:

$$\%\text{Ndfa} = \left(\frac{\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{reference}} - \text{B}} \right) \text{x}100 \quad (2)$$

where $\delta^{15}N_{reference}$ is the δ^{15}N value of the reference plant, B is the δ^{15}N of sulla deriving N only from N_2-fixation, and $\delta^{15}N_{legume}$ is the δ^{15}N value of the legume sample. As a reference plant we used Italian ryegrass, that is a non-nodulating plant, cropped under the same shading treatments of sulla on neighbouring plots. Sulla samples were matched to reference plants from the plot with the same shade level treatment and the same sampling date. The B value for sulla was found in the literature, from the study by Gentili et al. (2019) (-1.3 δ^{15}N). The fixed Nitrogen (Nfix) (g m^{-2} of N) was calculated as Nyield x (% Ndfa/100). Finally, the cumulative values of Nyield and Nfix were computed for each experiment (Experiment 1 and Experiment 2) by summing up the Nyield and Nfix values obtained in each plot across the two experimental years, respectively for shoots and roots. Shoot:Root Ratio (SRR) was calculated using the cumulative values of shoot and root Nyield and Nfix, respectively.

2.5. Statistical analysis

The statistical data analysis was performed using R software (R Core Team, 2021) to assess the effect of shading (S) on shoots and roots of sulla. In the shoot data analysis, the effect of S and mowing (M) on N%, δ^{15}N, %Ndfa, Nyield and Nfix was evaluated. The effect of S and M was investigated using the lmer() function for linear mixed-effect models of the "lme4" package (Bates et al., 2015) with factors S and M as fixed effects and replicate as random effect. The differences between factors and their interactions were assessed with a two-way analysis of variance (ANOVA). Because RYE did not produce aboveground biomass (AGB) during the November mowing in Experiment 1 (2020), it was not possible to evaluate the effect of shade treatments within each level of the model. Therefore, data related to this harvest time (M5), which were dependent on dry matter (DM) (%Ndfa and Nfix), were excluded from the statistical analysis.

The roots data were analyzed to investigate the effect of S and the year (Y) of sampling on the same parameters. The effect of S and Y was assessed using the lmer() function with factors S and Y as fixed effects and replicate as random effect. The differences between factors and their interactions were also assessed with a two-way analysis of variance (ANOVA).

Lastly, the effect of the shade treatments on cumulative values computed in terms of Nyield and Nfix on the SRR was assessed using the lm() function for linear models. The Shapiro–Wilk test was used to verify the normality of residuals, while Bartlett's test was used to verify homoscedasticity. Subsequently, Tukey's HSD post-hoc test was performed through pairwise multiple comparisons using the "emmeans" R package (Lenth et al., 2020) with the emmeans() function. Outliers were detected and removed using the Bonferroni outlier test with the outlierTest() function from the "car" package (Fox and Weisberg, 2019). Data transformation was not required for any of the dependent variables.

3. Results

3.1. Meteorological conditions

In the first year of the trial (2019–2020), cumulative rainfall from sowing (20 October 2019) to the final harvest (23 June 2020) totaled 822 mm, which was higher than the long-term average of 737 mm (Fig. 1). In 2020–2021 (2nd year of the Experiment 1 and 1st year of the Experiment 2), cumulative rainfall during the crops growing season, from November 2020 to June 2021 was closer to the long-term average (697 vs 626 mm, respectively). In the third year of the trial (2nd year of Experiment 2), cumulative rainfall during the growing season, from October 2021 to May 2022, was lower with respect to the long-term average (563 vs 697 mm, respectively). Across the three seasons of the study, rainfall distribution varied in autumn and winter, but were similar in spring (Fig. 1). The daily mean temperature during the growing season (November-June) was 13.6 °C in 2019–2020, 12.3 °C in 2020–2021 and 11.5 °C during the season 2021–2022 (November-May). The long-term mean temperature was lower (12.4 °C) compared to 2019–2020, similar to 2020–2021, and higher than 2021–2022 (Fig. 1).

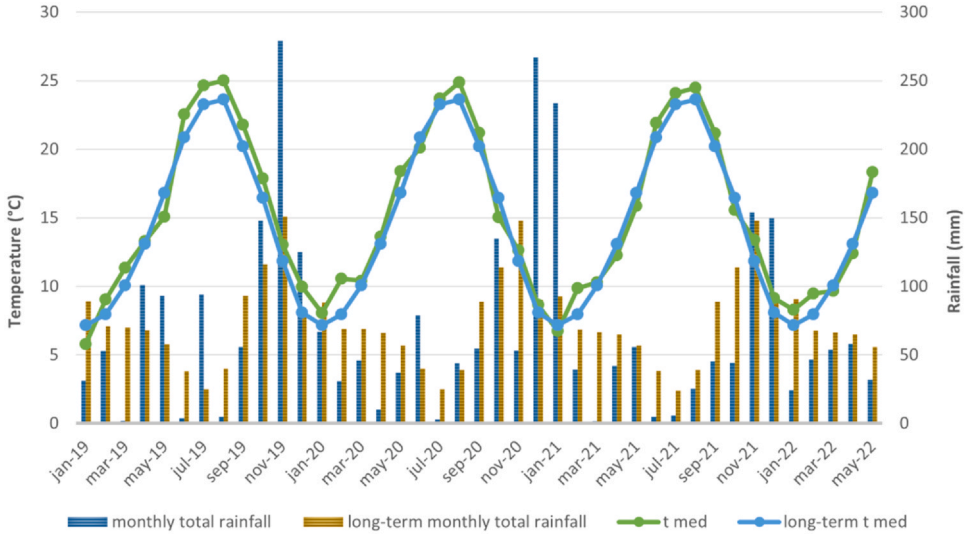

Fig. 1. October 2019–May 2022 and long-term (1993–2021) monthly total rainfall (mm) and mean air temperature (t med, °C), San Piero a Grado (Pisa, Italy).

3.2. Effects of light reduction on the content and the origin of N in shoots

3.2.1. Experiment 1 (2020-2021)

The statistical analysis showed that: (i) a significant interaction effect M x S was recorded only for the %Ndfa ($p \leq 0.01$); (ii) S significantly affected $\delta^{15}N$ ($p \leq 0.001$) and Nyield ($p \leq 0.001$) (Table S1).

The mean values, averaged over the seven mowings, showed how in the shading treatments HS and MS, the $\delta^{15}N$ (1.26 and 1.56 mg g^{-1}) and Nyield (3.33 and 3.69 g m^{-2} of N) were significantly lower than NS (2.37 mg g^{-1} and 5.08 g m^{-2} of N for $\delta^{15}N$ and Nyield, respectively) (Table S2). About Ntot, no differences occurred among the shading treatments (on average 3.33 %).

Fig. 2 shows significant differences %Ndfa per each mowing time (factor M) among the different levels of the factor S, through a pairwise multiple comparison of nested effects. For the Ntot, no clear tendency was observed among shading level and harvest times. The %Ndfa showed lower values for NS than shaded treatment, and in June 2020 (M4), it was significantly lower than MS and HS (25.84 vs 66.52 % and 79.33 %), which did not differ from each other.

3.2.2. Experiment 2 (2021-2022)

The statistical analysis showed that: (i) a significant interaction effect S x M was observed for Ntot, $\delta^{15}N$ and %Ndfa ($p \leq 0.05$); (ii) S affected Nyield ($p \leq 0.001$) and Nfix ($p \leq 0.01$) (Table S1).

The averaged values over the five mowings showed a significant effect of S on Nyield and Nfix. Specifically, their values at NS (7.37 and 5.22 g m^{-2} of N, respectively) were both significantly higher than those at HS (4.75 and 3.20 g m^{-2} of N) (Table S3).

Fig. 3 shows significant differences in Ntot, $\delta^{15}N$ and %Ndfa for each mowing time (factor M) among the different levels of the factor S, through a pairwise multiple comparison of nested effects. The Ntot showed lower values in NS plots at two harvest times during spring

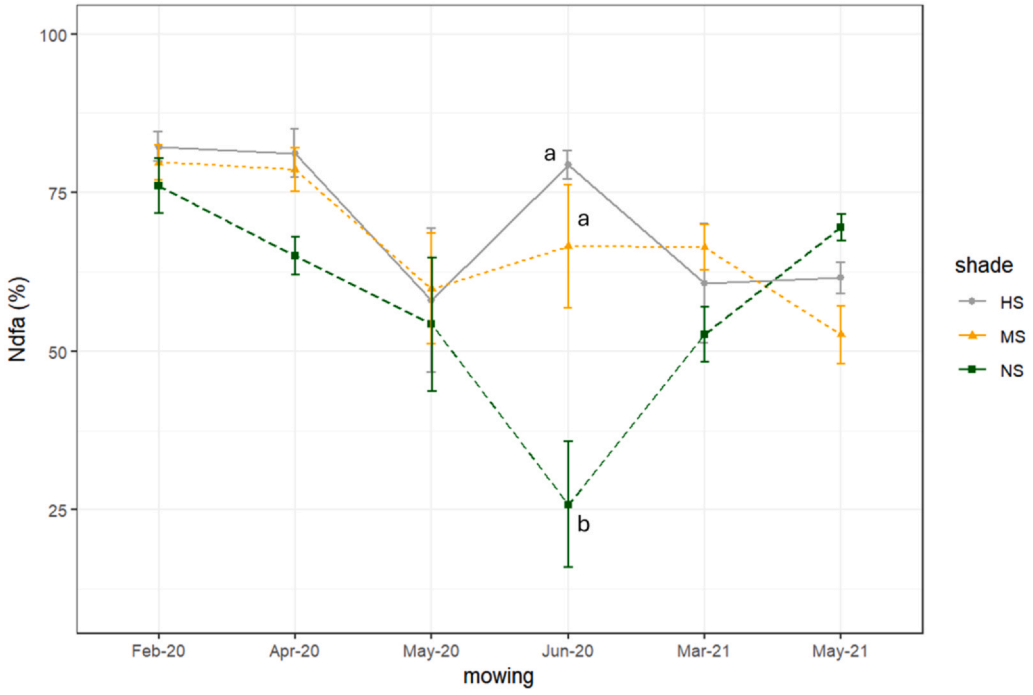

Fig. 2. Values of percentage of N derived from the atmosphere on total N content (%Ndfa), measured on shoots of sulla (cv. Silvan) at diverse levels of shade in each mowing in experiment 1: February 2020 (M1), April 2020 (M2), May 2020 (M3), June 2020 (M4), March 2021 (M6), May 2021 (M7). Vertical bars indicate standard error of the mean. Within each mowing where a significant effect of shade was observed, treatments with the same letter were not significantly different at $p \leq 0.05$ (Tukey's HSD test). NS means no shade, MS mean 30 % of shade, and HS means 50 % of shade.

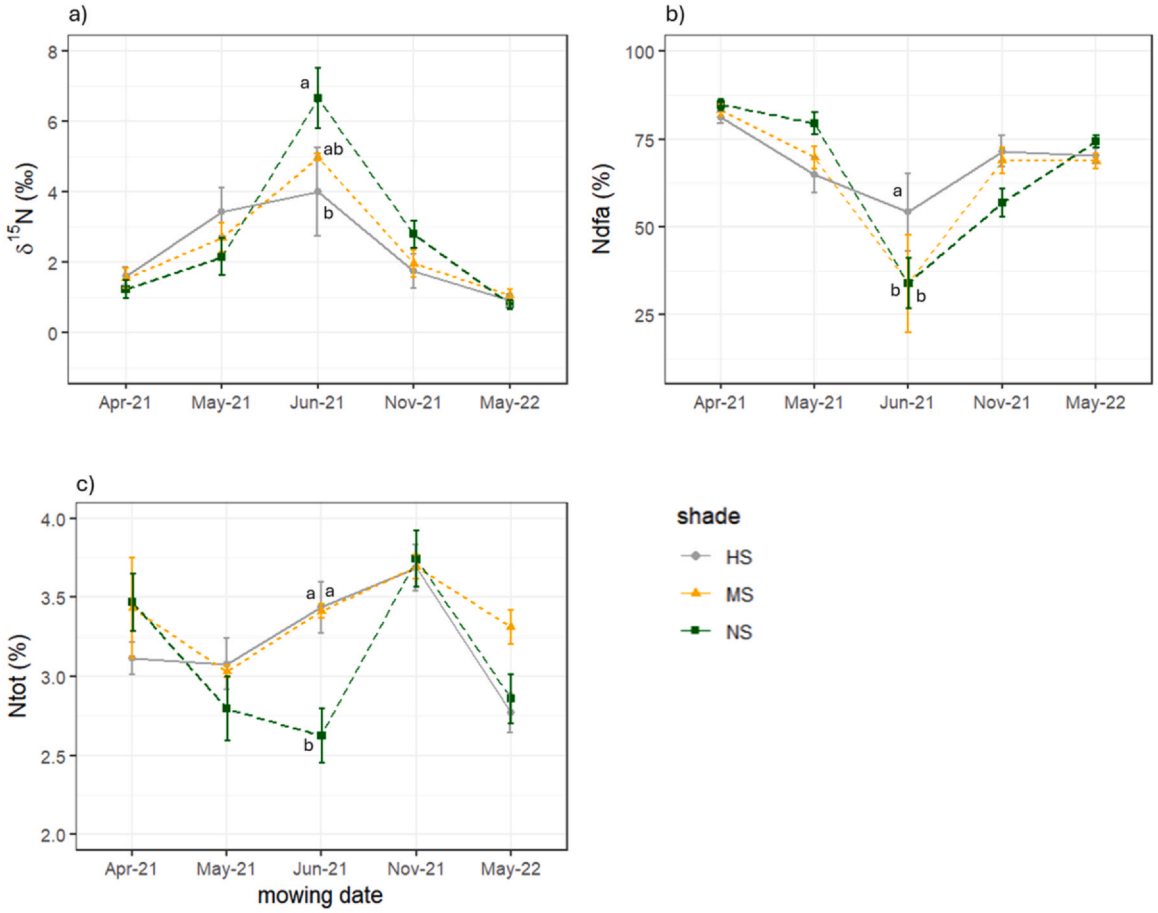

Fig. 3. Values of (**a**) ^{15}N atom excess respect to the atmosphere ($\delta^{15}N$), (**b**) percentage of N derived from the atmosphere on total N content (%Ndfa), (**c**) total N concentration (Ntot), measured on shoots of sulla (cv. Silvan) at diverse levels of shade in each mowing in experiment 2: April 2021 (M1), May 2021 (M2) and June 2021 (M3), November 2021 (M4), May 2022 (M5). Vertical bars indicate standard error of the mean. Within each mowing where a significant effect of shade was observed, treatments with the same letter were not significantly different at $p \leq 0.05$ (Tukey's HSD test). NS means no shade, MS mean 30 % of shade, and HS means 50 % of shade.

2021; in particular, in June 2021 (M3) total N concentration in shoots was significantly lower in NS than MS and HS (2.62 vs averagely 3.40 % for MS and HS plots) (Table S3), but MS and HS did not differ to each other. $\delta^{15}N$ and %Ndfa showed significant differences among S treatments only at M3 (May 2021). $\delta^{15}N$ showed an increase at NS in comparison to HS (6.65 vs 3.99 mg g^{-1}), but no difference was

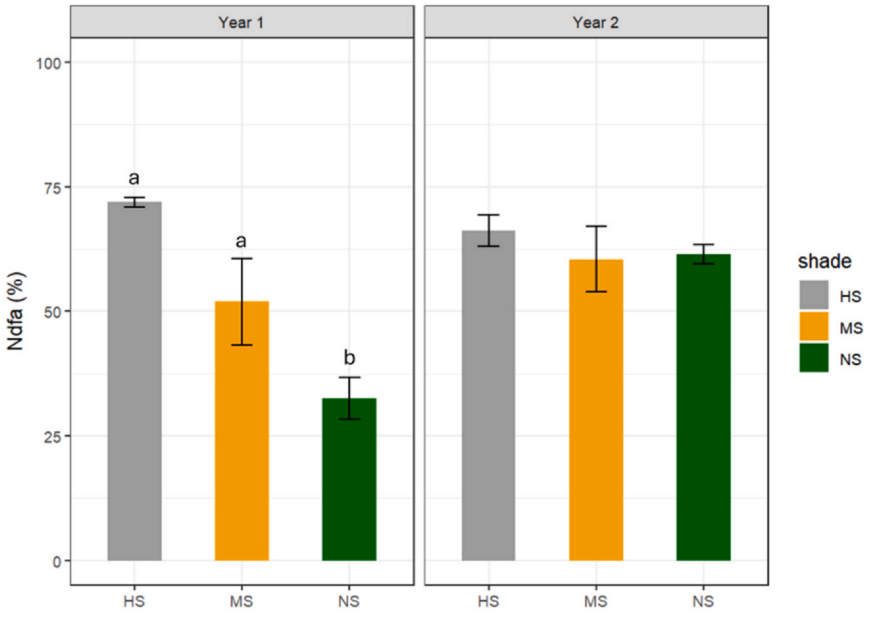

Fig. 4. Values of N derived from the atmosphere on total N content (%Ndfa) measured on roots of sulla (cv. Silvan) at diverse levels of shade, in each year of sampling (Year 1, i.e., September 2021, and Year 2, i.e. September 2022) in experiment 2. Vertical bars indicate standard error of the mean. Within each year, where a significant effect of shade was observed, treatments with the same letter were not significantly different at ($p \leq 0.05$ Tukey's HSD test). NS means no shade, MS mean 30 % of shade, and HS means 50 % of shade.

observed considering MS respect to NS and HS Consequently, %Ndfa in NS and MS plots was lower than HS (34.03 and 33.85 vs 54.24 %).

3.3. Effects of light reduction on content and origin of N in roots

3.3.1. Experiment 1 (2020-2021)

The statistical analysis on root samples showed that: (i) no significant interaction effect of S x Y was observed for any of the studied variables, (ii) S did not affect all parameters, (iii) while all parameters, except %Ndfa, were affected by year (Y) of sampling (Table S4).

Averaged values over the two years of sampling showed the same trend observed for shoots but without any statistical differences. The % Ndfa showed a trend towards higher values in both the shading treatments (on average, 71.50 %) compared to NS (51.73 %), whereas we observed an opposite trend for the Nyield, with NS showing higher values than HS (3.38 vs 1.83 g m^{-2} of N). Considering the effect of the year, N (%), $\delta^{15}N$, Nyield and Nfix were significantly higher in the first year of sampling (Table S5).

3.3.2. Experiment 2 (2021-2022)

In Experiment 2, different results in respect to Experiment 1 were recorded for the content and origin of N in the root biomass of sulla. The statistical analysis showed that: (i) a significant interaction effect S x Y was observed only for %Ndfa ($p \leq 0.01$); (ii) averaged over years, S affected $\delta^{15}N$ ($p \leq 0.05$), (iii) all parameters, except Ntot, were affected by Y (Table S4).

With regard to $\delta^{15}N$, the NS induced significantly higher values than HS (3.56 vs 1.67 mg g^{-1}), but no difference was observed for both when compared to MS. Furthermore, considering the effect of the year averaged over the shade levels, $\delta^{15}N$ was significantly higher in the first year (3.59 vs 1.72 mg g^{-1}), whereas, in contrast, Nyield and Nfix showed significantly higher values in the second year (1.63 vs 3.80 and 0.91 vs 2.35 g m^{-2} of N, respectively). (Table S6).

Fig. 4 shows significant differences among the S treatments for % Ndfa in each of the two years of sampling, through a pairwise multiple comparison of nested effects. In the first year, the percentage of Ndfa showed significant differences, indeed NS was lower than MS and HS (32.50 vs 51.90 % and 71.92 % respectively), while no differences were observed in the second year.

3.4. Cumulative values of N yield and amount of N derived from N_2-fixation in shoots and roots

Both Nyield and Nfix in shoots and roots of sulla were weakly affected by S. Indeed, S only significantly affected shoot Nyield and Nfix in Experiment 2 ($p \leq 0.01$ and $p \leq 0.05$, respectively) (Table S7).

As shown in Table 1, in both Experiments we observed a negative trend in Nyield and Nfix in shoots at increasing levels of shade. For Experiment 2, NS resulted in significantly higher Nyield and Nfix values than HS, with MS not being statistically different from the other treatments. For roots, a different trend was observed in the two Experiments. In Experiment 1, similar values of Nyield and Nfix were shown by NS and MS, which were both slightly higher cumulative Nyield and Nfix than HS. In Experiment 2 we did not notice a clear trend for both variables. Furthermore, the statistical analysis of data obtained from the two experiments revealed that S did not affect the shoot:root ratio calculated on total N yield and neither on the total mass of N derived from N_2-fixation (Table S8).

As shown in Fig. 5, Although different trends in shoot:root ratio were shown between the experiments, Nyield and Nfix followed similar trends in the two experiments. In Experiment 1 there was a trend towards slightly higher Nyield and Nfix values in HS, followed by NS and then MS. In contrast, in Experiment 2, Nyield and Nfix followed a decreasing trend at increasing levels of shade (Table S9).

4. Discussion

The aim of this study was to assess the effect of increasing levels of artificial shade on BNF of sulla. The field trial was carried out in rainfed conditions on a soil with a medium-low content of total N (1.29 g kg^{-1} of N, as average of the two fields). Generally, the range of sulla %Ndfa values found in this experiment was comparable to those reported in the literature for subtropical conditions (Bell et al., 2017) and Mediterranean rainfed conditions (Sulas et al., 2009).

In this study, the total N concentration (Ntot) in shoot biomass was not affected by shading in Experiment 1, while in Experiment 2 we observed increasing values in the shaded plots (i.e., MS and HS). This trend was particularly evident in the May sampling (2.62 % in NS vs averagely 3.40 % for MS and HS) when the light intensity is a limiting factor for cool-season forage production, as observed in a previous work from the same experiment (Tramacere et al., 2024). These results were confirmed by Sanna et al. (2019) who demonstrated a significant increase in shoot nitrogen percentage in legume-based swards under partial shade. Carranca et al. (2015) also observed that tree canopies influenced BNF by pasture legumes in *Montado* silvopastoral systems in Portugal. Their findings showed higher N concentrations in forage plants grown beneath the tree canopy compared to those grown outside. Unlike our study, which was based on slats and artificial shade, the detrimental effect of trees on nitrogen concentration in forage crops in silvopastoral fields could be due not only to light limitation, but also to other forms of belowground competition between plants.

Regarding the average effect of shade on $\delta^{15}N$, in our study we observed different results in the two experiments: (i) in Experiment 1 significantly higher values for $\delta^{15}N$ in NS plots were observed for shoots, while (ii) in Experiment 2 shade did not significantly affect this parameter. The reason for this variable effect might be found in the diverse weather conditions in the establishment year of the plot field. Interestingly, in both experiments in June samplings (M4 and M3 respectively for Experiment 1 and Experiment 2), when the temperature is high and rhizobial activity in full sun could be lower, the lowest nitrogen fixation percentage was observed in shoots of sulla grown on the full sun plots (NS), while shade plots maintained elevated % of N-

Table 1
Cumulative average values (g m^{-2} of N) of total N content (Nyield) and content of N derived from N_2-fixation in mass units (Nfix), measured on shoots and roots of sulla (cv. Silvan) at different levels of shade in each experiment (i.e., Experiment 1 and Experiment 2). In each experiment, treatments sharing the same letter did not show significant differences at $p \leq 0.05$ (Tukey's HSD test). NS means no shade, MS mean 30 % of shade, and HS means 50 % of shade.

Nitrogen cumulative value (g m^{-2} of N)		Shoot		Root	
Experiment	Level of shade	Nyield	Nfix	Nyield	Nfix
1	HS	22.62 ± 4.44	15.52 ± 2.80	3.67 ± 0.99	2.66 ± 0.77
	MS	25.88 ± 2.69	15.33 ± 2.04	6.09 ± 1.27	4.08 ± 0.57
	NS	34.91 ± 3.00	19.29 ± 0.91	6.77 ± 1.06	3.79 ± 0.82
2	HS	23.76 ± 1.86 b	16.58 ± 1.35 b	6.03 ± 1.13	3.78 ± 1.00
	MS	31.12 ± 2.49 ab	21.69 ± 1.01 ab	5.37 ± 1.11	2.78 ± 0.82
	NS	36.85 ± 1.46 a	26.13 ± 1.80 a	7.02 ± 3.26	3.04 ± 0.66

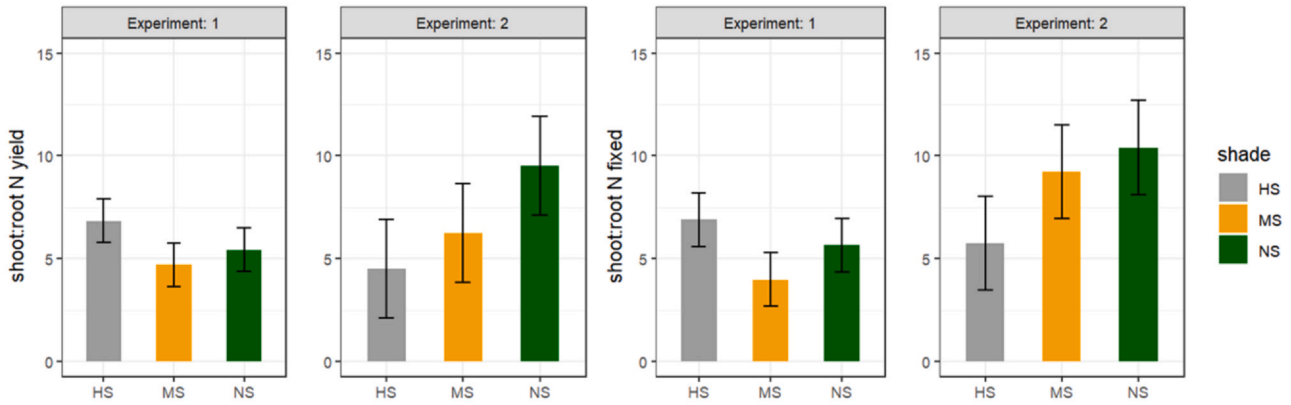

Fig. 5. Values of shoot:root ratios for cumulative total N content (Nyield) and cumulative content of N derived from N_2-fixation in mass units (Nfix), measured on sulla (cv. Silvan) at diverse levels of shade, in each experiment (i.e., Experiment 1 and Experiment 2). Vertical bars indicate standard error of the mean. Within each experiment, no significant effect of shade was observed ($p \leq 0.05$ Tukey's HSD test).

fixation (Figs. 2 and 3). Moreover, in Experiment 2, roots showed similar results to shoots in the Experiment 1, with an increased percentage of N derived from N_2-fixation in HS than in NS plots in the establishment year. The study of Querné et al. (2017) confirmed our results obtained in the Experiment 1; indeed, their results from an alley cropping trial in the south of France, where 17-year-old hybrid walnut (*Juglans nigra* × *regia* cv NG23) trees were intercropped with alfalfa (*Medicago sativa* L.), challenged the common assumption that N fixation diminished in the shade of trees. In fact, Querné et al. (2017) indicated that BNF was stimulated near the trees, implying the presence of compensatory and facilitative mechanisms between the non-legume trees and the understory legumes (e.g., the reduced N availability due to tree root competition). In our study, the higher %Ndfa in the shaded plots than in NS observed in the Experiment 1 was likely due to a compensation effect for the lower yields of sulla in shaded plots (data on sulla yield reported by Tramacere et al., 2024). Overall, the effect of this compensation was only a slight reduction in the content of fixed N (Nfix) in shaded plots in respect to NS, as shown also by the cumulative values. On the other hand, results from the study of Goh et al. (1996), conducted in a *Pinus* radiata D.Don. AFS in a sub-humid environment of New Zealand, showed that the distance of the sward in respect to the trees did not significantly affect the %Ndfa of the legumes, indicating that trees had little effect on N fixation, but rather on pasture growth. Indeed, although our findings from Experiment 2 showed that the amount of N fixed was higher at NS, the %Ndfa was not affected by shade. By contrast, Isaac et al. (2014) found that averaged over all the tree species investigated, soybean (*Glicine max* (L.) Merr.) %Ndfa, foliar Ntot and content of N from N_2-fixation were higher in intercropping than in monocropping. The authors hypothesized the possible stimulation of rhizobial activity in soybean grown together with trees, due to belowground interspecies competition for resources. As soybean showed better results with distance from the trees, where the competitive effects were reduced, the authors argued that the aforementioned interactions might have reached excessive levels near the tree trunk. Studying N dynamics in chickpea (*Cicer arietinum* L.) alley cropped with walnut trees, Mahieu et al. (2016) found that the %Ndfa in whole chickpea plants was only half of that observed in sole crop plots. However, the results also indicated that environmental conditions in the middle of the alley (particularly, the cumulative radiation at 90 % of full sunlight), were advantageous for chickpea seed yield. This benefit was likely due to increased soil mineral N availability and moisture caused by the absence of trees.

Considering the overall Nyield of shoots, we observed in Experiment 1 for both MS and HS lower Nyield with respect to the plots under full sun light (3.33 and 3.69 vs 5.08 g m^{-2} of N, respectively). Likewise, in Experiment 2, the Nyield was lower in the shaded plots, but only for the HS treatment. The cumulative values showed a significant effect of the HS to reduce N yield only in Experiment 2, confirming that especially intensive shade may affect the Nyield of the herbage biomass. Considering cumulative values for roots, no clear tendency was highlighted. The study of Goh et al. (1996) partially confirmed our results; indeed, the authors found that, overall, trees did not significantly impact the annual total Nyield of the pasture. However, they observed that pastures located north of the trees exhibited significantly higher dry biomass and Nyield in early spring compared to those in other positions. The results of Yunusa et al. (1995) from the same trial suggested that the different response of forage species at different relative positions respect to the trees was due to different levels of interception of total radiation and different soil temperature. These two environmental parameters might have played a crucial role affecting plant biomass production and N yield in a season in which these parameters were limiting for the growth of the crop. These results highlight the importance to study not only the mean or cumulative effect of shade on herbage production and N content, but rather to focus much more on key seasonal and crop phenological stages.

Concerning the Nyield and Nfix shoot:root ratio in our work, the results from the two experiments showed no significant effect of shade and different average trends (although the tendence was similar for Nyield and Nfix). For these parameters, the allocation dynamics of the resources within plants may play a role that likely depends upon the weather conditions and the health status of the plant during the vegetative season. Therefore, explaining these data was complicated, especially considering the different trends observed.

To the best of our knowledge, comparing our results with N fixation performances of other annual and perennial legumes grown under different levels of shade is not feasible due to the scarcity of studies in southern European Mediterranean areas. However, results from our study highlighted that while the %Ndfa was higher in the shaded plots, the total N yield, primarily dependent on shoot biomass production, was generally higher in the non-shaded plots. This could suggest that sulla in shaded plots might allocate fewer resources (i.e., photosynthates) to shoot biomass production, while investing more energy in accumulating reserves in the roots, potentially enhancing rhizobial activity due to the increased resource availability. Understanding these dynamics is important to identify the optimal conditions for various agrosilvopastoral systems, to optimize all nutrient pathways mediated by plants for effective agroecological management and enhanced agroecosystem service delivery.

5. Conclusion

Legumes are considered crucial in strategies for increasing production and sustainability in Mediterranean AFS by reducing soil fertility degradation and by providing N to the soils, thereby enhancing tree

growth. Therefore, it is key to assess which factors might negatively affect symbiotic N_2 fixation and to manage or avoid these factors. Additionally, selecting the appropriate legume species is crucial. For example, sulla ensures long-term production suitable for extensive grazing, but little is known about its adaptability to the shade conditions typical of AFS. The purpose of this study was to measure the N fixation dynamics of sulla grown under different levels of shading (i.e., 0 %, 30 % and 50 % of light reduction respectively), and to verify its suitability for utilization in AFS through N_2-fixation assessment. Although the percentage of N derived from N_2-fixation was higher in the shaded plots, the total N yield, which depends mainly on shoot biomass production, was generally higher in non-shaded plots. Hence, despite a potential reduction in biomass production and N yield, sulla seems to be a suitable forage for introduction into AFS, given its increased capacity to fix atmospheric nitrogen under shaded conditions and store it in a stable form in the soil, which could potentially be exploited by trees. We demonstrated that the use of artificial shade equipment in combination with the analysis of the ^{15}N natural abundance technique for N_2-fixation assessment could be an effective strategy to conduct extensive, cost-effective and time-saving studies on different forage species and even their genotypes. However, it is highly recommended to also perform field studies under real agroforestry conditions to optimize the introduction of legumes in AFS, as other facilitative and competitive interactions might occur in real AFS with cascading effects on N_2-fixation. In fact, in our study, we simulated the shadow provided by trees, but with the awareness that we cannot consider the effect of tree roots, especially what happens in the tree's close proximity. Further studies are needed to better understand the ecophysiological relations between N_2-fixation and shading in AFS, also considering microclimate conditions. An in depth and environment-specific analysis at soil microbiome level might be also useful to unravel the microbial mechanisms underpinning the different trends shown in the literature. Finally, it would be interesting also to assess if the N fixed by legumes could be transferred to the trees.

CRediT authorship contribution statement

Antichi Daniele: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Ragaglini Giorgio:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Mele Marcello:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Mantino Alberto:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Tramacere Lorenzo Gabriele:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data availability

The data that support the findings of this study are available in the Zenodo open access repository at this link: https://zenodo.org/records/13306844.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ijagro.2025.100028.

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